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Diagnostic challenges of today's lung cancer pathology: personalizing therapy by immunohistochemical and molecular biomarkers

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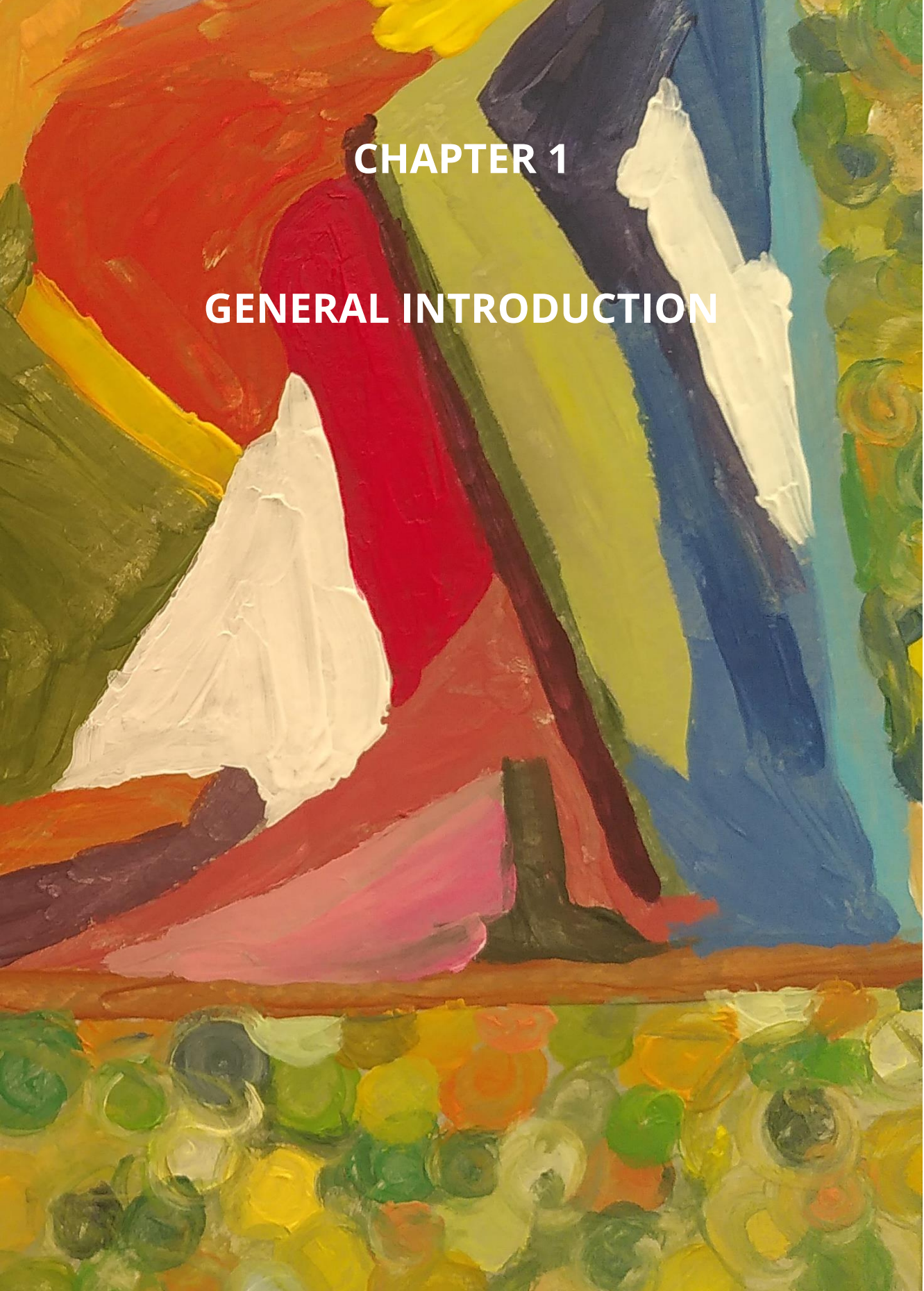

CHAPTER 1

GENERAL INTRODUCTION

Chapter 1: General introduction

1.1 Case presentation, a patient journey anno 2023

A 62-year old former smoker visits the pulmonologist because a pulmonary node in the left upper lobe is discovered. His symptoms are limited, only a dry cough that he has had for a few weeks. A CT-guided biopsy is taken, which leads to the diagnosis of non-small cell lung cancer (NSCLC). Specifically a TTF-1 positive adenocarcinoma with lepidic, papillary and micropapillary growth patterns is diagnosed. After comprehensive staging with PET-CT and EBUS, it's established that the patient has a T1bN0M0 tumor. He undergoes a lobectomy of the left upper lobe. The resection margins are tumor-free and the carcinoma has not infiltrated the visceral pleura.

After 2 years and 3 months, a liver nodule is revealed, and a biopsy confirms that it is a metastasis of the prior lung adenocarcinoma. Thus, the patient is now stage IV, which warrants additional molecular and immunohistochemical tests. The PD-L1 tumor proportion score is 5% and mutations in EGFR p. L858R and TP53 p. V157S are identified with DNA NGS. The patient is treated with Osimertinib. Following an 18-month period of stable disease, growing lesions are discovered in the adrenal gland an lymph nodes. A new biopsy is taken from one of the growing mediastinal lymph nodes via fine needle aspiration, in which an EML4:ALK fusion is identified, in addition to the EGFR p. L858R and TP53 p. V157S mutations. The patient is treated with chemoradiation and dies within 9 months. (Figure 1)

This case, of which there are hundreds of similar ones in the Netherlands each year, illustrates the complexity of the current NSCLC patient journey. The pathologist is prominently involved, and is required to assess the case at key decision-making moments in the disease process: at the early-stage diagnosis, at the late-stage diagnosis and at the moment of acquired resistance.

1.2 Introduction Outline

In this introduction, the characteristics of NSCLC, including the molecular makeup and genomic heterogeneity are comprehensively addressed. The most important novel treatments are outlined: targeted tyrosine kinase inhibitors (TKIs) and immune-checkpoint inhibitors (immunotherapy), including a

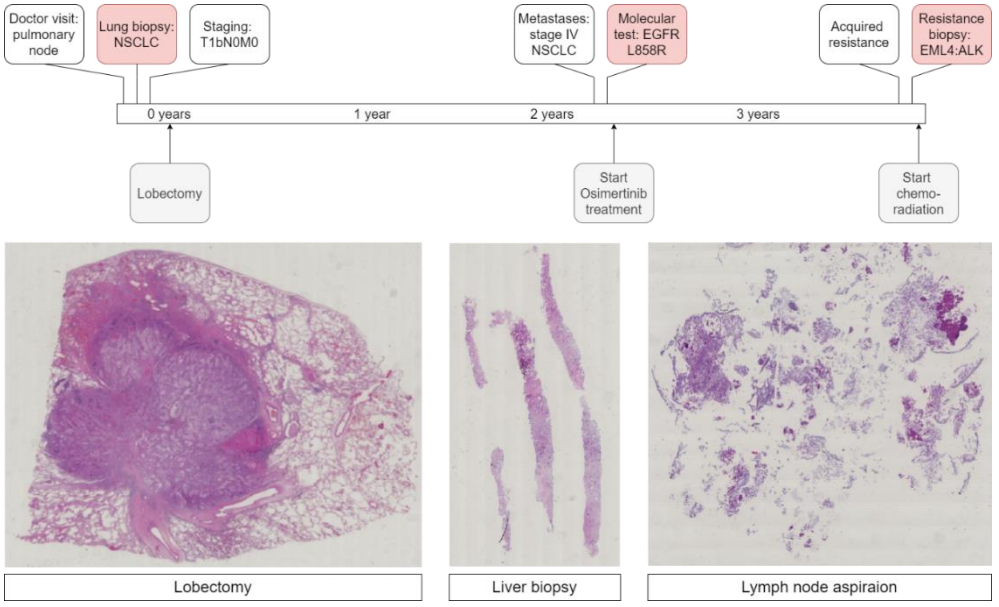

***Figure 1**: Case patient journey. Red: pathologist tasks. Grey: New treatment.*

detailed description of testing techniques to select patients for either of these therapies. In the final paragraph, the societal impact of lung cancer research will be discussed.

1.3 Lung Cancer demographics

Lung cancers are one of the most common and deadly cancers worldwide, with 1.6 million deaths annually. [1] In the Netherlands, approximately 13,000 new lung cancer patients are diagnosed each year, the majority of which suffer from non-small cell lung cancer (NSCLC). [2]

The high death rate of NSCLC is in part due to the late stage at diagnosis: due to the localization, many tumors remain asymptomatic until after the tumor has metastasized. Approximately 50% of patients are therefore diagnosed in stage IIIB or IV. In addition, early stage tumors are not always successfully cured. Approximately 50% of patients who undergo surgical resection die of lung cancer within 5 years, likely due to the presence of occult metastasis at the time of surgery.

NSCLC can be divided into two main subtypes: lung adenocarcinoma and lung squamous cell carcinoma. [3, 4] Adenocarcinomas can be further divided using growth patterns or differentiation grade, leading to a stratification of patients in low risk (well differentiated, lepidic growth), intermediate risk (moderately

differentiated, papillary or acinar growth) and high risk (poorly differentiated, solid, complex glandular or micropapillary growth). [5]

Up to 90% of lung cancers can be attributed to tobacco smoking. [6] In 2019, 21.7% of adults and 8% of children aged 12-16 in the Netherlands were smokers. [7] Worldwide, 20% of individuals aged 15 or above are smokers. (Figure 2) In 2019, 7.7 million deaths were attributable to tobacco smoking worldwide, making smoking the cause of 13.6% of all deaths that year. [8] In the Netherlands, 13.1% of deaths are still attributed to smoking in 2019, which is only just below the global average.

Smoking is more common in people in a lower socio-economic class, making tobacco addiction a true poverty disease. In the Netherlands, 15.4% of HBO and University-educated people were smokers, versus 26.2% of people who attended VMBO, MAVO, LBO or primary school only. [7] Remarkably, although smoking prevalence is slowly decreasing in the Netherlands, the number of smokers is still rising in many other countries, which are often low- to middle-income countries: Azerbaijan, Georgia, Kyrgyzstan, Mongolia, Uzbekistan, Albania, Bosnia and Herzegovina, North Macedonia, Serbia, Belarus, Lithuania, Moldova, Russia, Antigua and Barbuda, Belize, Grenada, El Salvador, Afghanistan, Egypt, Iran, Jordan, Lebanon, Saudi Arabia, Federated States of Micronesia, Kiribati, Solomon Islands, Tuvalu, Indonesia, Laos, Congo, Equatorial Guinea, Gabon, Djibouti, Lesotho, Côte d'Ivoire, Guinea-Bissau, Mali, Niger and São Tomé and Príncipe. [8]

Therefore, perhaps the most important aspect of lung cancer management is prevention. Smoking eradication, together with screening (heavy) smokers for pulmonary nodules is paramount. It was recently demonstrated that low-dose CT screening in heavy smokers aged 50-74 reduces lung cancer related mortality by 24%, which is an important argument in favor of population screening. [9]

1.4 Molecular landscape of NSCLC; smokers versus non-smokers

The vast majority of NSCLCs are caused by the inhalation of carcinogens (tobacco smoke, air pollution and occupational carcinogens). These carcinogens lead to an accumulation of DNA-mutations, which drives cells to

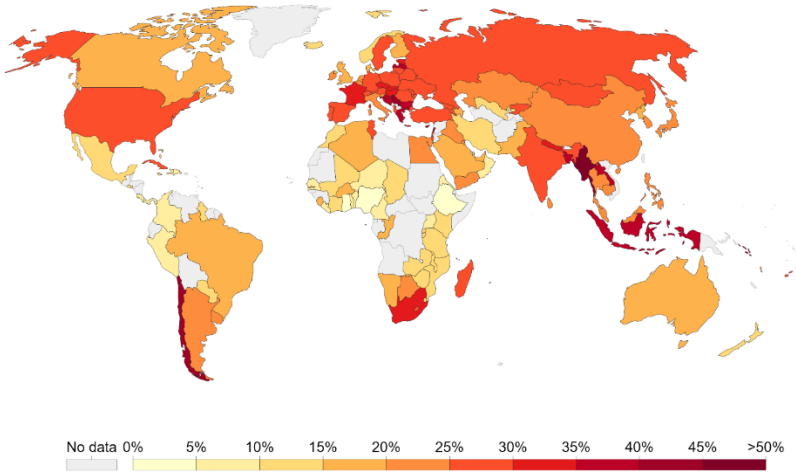

***Figure 2**: Worldwide prevalence of smoking in 2018, measured in individuals aged 15 and older. Adapted from Ritchie et al, Our World In Data.* [10]

malignant behavior via the alteration oncogenic driver genes (such as KRAS and EGFR) or tumor-suppressor genes (such as TP53 and CTNNB1). [3, 4, 11] Due to this mechanism of carcinogenesis, tobacco-related lung cancers can harbor many different DNA-alterations and are highly heterogeneous in their molecular makeup. [3, 12] Typical smoking-related DNA-alterations are KRAS, BRAF, PTEN, PIK3CA and TP53. [13]

A minority of NSCLCs arises in never-smokers. These tumors have a different molecular signature and more frequently harbor mutations in ALK, ROS1, RET, HER2 and EGFR. [3, 13, 14] Tumors in never-smokers generally have a lower tumor mutational burden (TMB) and fewer co-mutations in tumor suppressor genes such as TP53. [13, 14] Never-smokers respond differently to treatment with TKIs, [15] immunotherapy [16] and chemotherapy. [17]

When assessing the smokers and never-smokers together, it's clear that NSCLC is a highly heterogeneous disease, both in the clinical and genomic aspect. Known driver alterations, their prevalence and common NSCLC growth patterns are outlined in Figure 3.

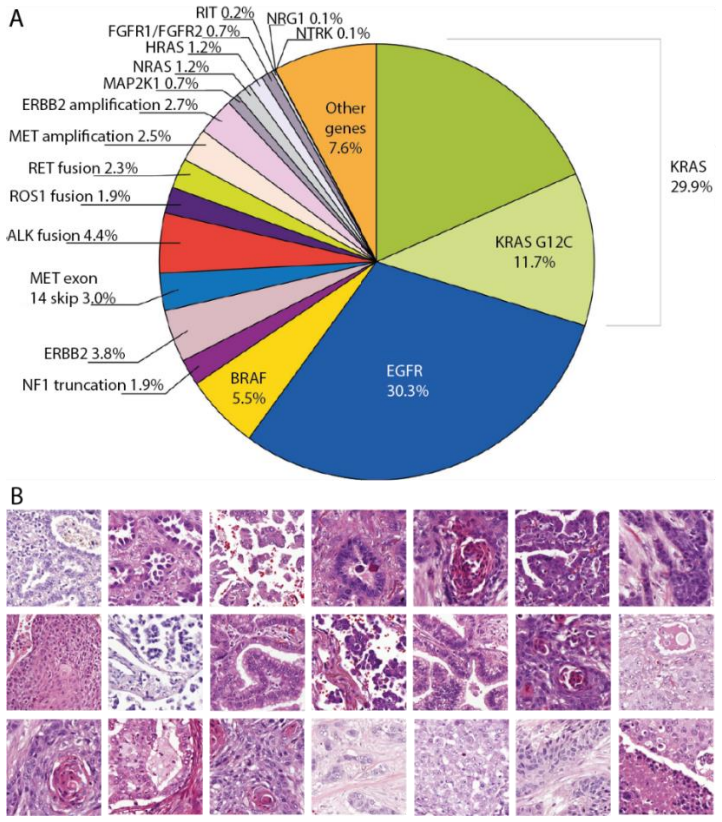

***Figure 3**: Lung cancer heterogeneity. A: oncogenic driver mutations in metastatic lung adenocarcinoma. Adapted from Addeo et al. [18] B: morphological heterogeneity in surgical NSCLC specimens.*

1.4.1 Intratumor genomic heterogeneity and tumor evolution

In addition to these differences *between* tumors, there is substantial genetic heterogeneity *within* tumors as well. Recent studies have reported that genomic aberrations often occur only in subclonal portions of lung cancer cells, which is called 'intratumor genomic heterogeneity'. This is observed for all mutations, including oncogenic drivers. [19, 20] Using multi-region tissue sequencing experiments, a distinction can be made between 'trunk mutations', that are homogenously present in all tumor sequencing regions, 'branch mutations', that are only present in part of the tumor sequencing regions, and germline mutations, present in all tumor and benign sequencing regions. (Figure 4) [20] It's hypothesized that intratumor genomic heterogeneity could

be the substrate for mixed response, acquired resistance, morphologic heterogeneity and tumor progression. [19]

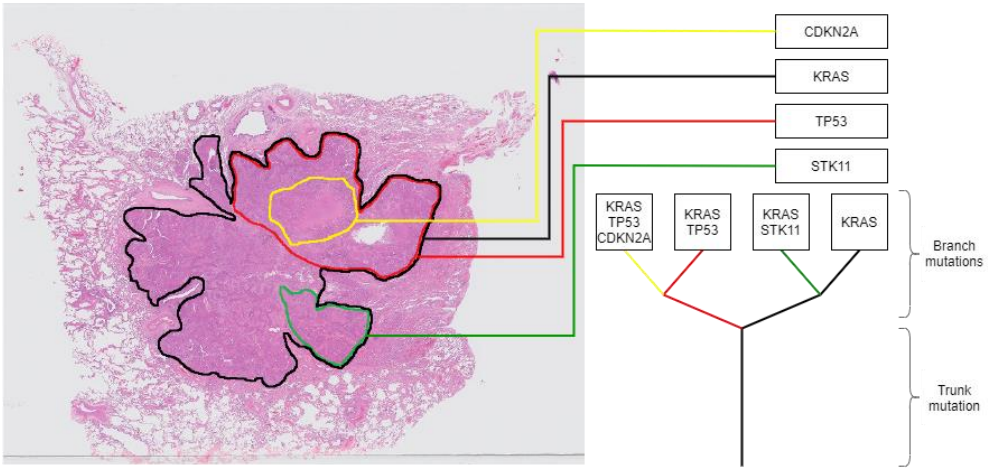

***Figure 4**: Example of intratumor genetic heterogeneity (ITH), with one trunk mutations (in this case: KRAS) and multiple branch mutations (in this case: STK11, TP53, CDKN2A).*

1.5 Targeted therapy and acquired resistance

For decades, the treatment of NSCLC was limited to surgery, radiation and chemotherapy, with limited survival benefit and substantial co-morbidity and mortality. [3, 4, 21, 22] An important change started in 2003, with the registration of gefitinib for chemotherapy-refractory metastatic NSCLC.

1.5.1 History: Gefitinib and other TKIs

Gefitinib, an antagonist for the endothelial growth factor receptor (EGFR), was first intended as a generic cancer growth inhibitor, but its introduction in the clinic in 2003 led to unexpected results. Whereas a small portion of chemotherapy-refractory NSCLC patients (10-19%) showed remarkable response to gefitinib, no effect was observed at all in the majority of patients. Later, in 2004, activating EGFR-mutations, such as L858R and exon 19 deletion, were identified in gefitinib-sensitive patients and showed to be absent in gefitinib-insensitive patients. [23]

These data formed the basis of a new and optimistic field of cancer research – targeted DNA-therapy. Simply finding the tumor's driver mutation and

inhibiting it with a specific antagonist – the cancer's kryptonite – would be the key to achieve durable response or even curation for all lung cancer patients.

In addition to EGFR, there are now tyrosine kinase inhibitors (TKIs) routinely available, targeting alterations in BRAF V600, [24] ALK, [25] MET, [26] HER2, [27] ROS1, [28] NTRK [29] and RET [30]. (Figure 5) For many other targets, such as BRAF non-V600, KRAS G12C and NRG1, new TKIs are available in the experimental context or via compassionate use or early access programs. However, for some targets, it has been difficult to find an effective TKI, and not all TKIs are perhaps as effective as we hoped: although progression-free survival has become significantly longer for stage IV patients with targetable mutations, complete curation is still far away.

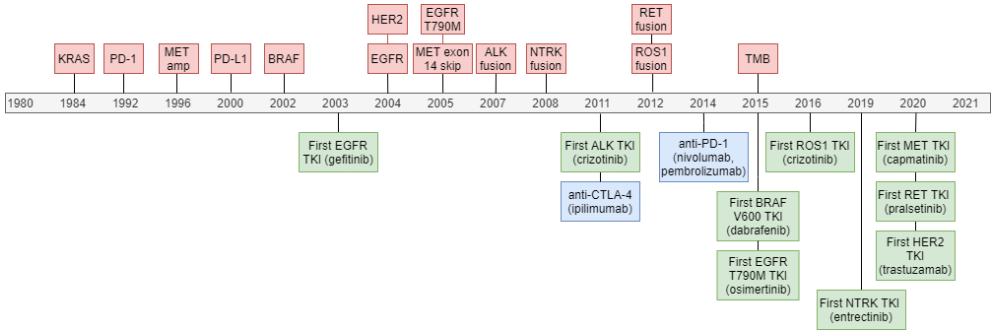

Figure 5*: timeline novel targets and therapies NSCLC. Red: Targets. Green: TKIs. Blue: immunotherapy.*

1.5.2 Acquired resistance

It has also become clear that, although many patients respond excellent to TKIs, eventually tumors bypass these inhibitory drugs and become resistant, often by acquiring additional oncogenic mutations. From that moment, one or more lesions will continue to grow and the disease progresses. [31-33] The mechanism by which the tumor acquires resistance can, in turn, be a targetable genomic aberration (such as EML4:ALK fusion), for which yet another TKI might be administered. [34]

A famous example is, again, EGFR. After the early successes of gefitinib and the discovery of mutations in the EGFR gene, the resistance mechanism EGFR p. T790M was discovered in 2005. The T790M mutation makes it impossible for gefitinib, afatinib and erlotinib to bind to EGFR, and prohibits its antagonistic effects. T790M therefore leads to reactivation of mutant EGFR and perfectly

explains the acquired resistance phenotype. In addition to T790M, many other resistance mechanisms have been discovered since, in part oncogenic driver mutations that we know, such as KRAS p. G12C, ALK fusion, HER2 amplification and MET amplification, but also novel mutations, such as EGFR p. C797S after treatment with Osimertinib. [35] Treatment with other TKIs, such as crizotinib, seems to follow the same principles as EGFR TKI treatment. [32, 36]

1.5.3 NTRK

An especially interesting, relatively novel target is NTRK. For most genetic targets, TKIs are first established in one cancer type and then translated to others. This is true for HER2, which was first discovered and treated in breast cancers; BRAF V600E, which was first discovered and treated in melanomas, and RET fusions, which were first discovered and treated in thyroid carcinomas. This tissue-oriented approach is a distinct disadvantage for patients with rare cancers, who are often the last to benefit from these new treatments.

Fusions in NTRK are extremely rare in NSCLC and in most cancers, whereas they are common in others (e.g. secretory carcinoma). Entrectinib and Larotrectinib trials therefore included all NTRK-rearranged metastatic solid tumors from the start, which has led to the registration of NTRK TKIs as first-line therapy for all solid tumors. [29, 37] This registration clearly marks the beginning of a shift from diagnosis-based to gene-based treatment.

1.6 Towards personalized medicine: selecting the right patients

There are many methods available for TKI and immunotherapy susceptibility testing, and finding the most optimal sequence can certainly be challenging. Often, there are significant time constraints, as the performance state of patients can be poor and waiting is not optimal, especially when brain metastases are present. In addition, the tissue available for genetic testing is rarely abundant; in some cases only cytology or small biopsy specimens are available, holding a limited number of tumor cells for molecular testing. When the tissue runs out before a definitive diagnosis is established, the patient has to undergo another biopsy, which can cause serious side effects and delay the diagnosis. Additionally, there can be financial limitations as well, since comprehensive genetic testing is costly. The pathologist therefore has to

identify the diagnosis method that minimizes time, tissue and expenses, while still achieving high effectiveness in finding targetable DNA-mutations.

As outlined in the patient journey figure (Figure 1), testing to select patients for immunotherapy or TKI treatment is now required at two specific moments: right after the diagnosis of metastatic lung cancer (1) and when acquired resistance occurs (2). In the future, testing will be implemented in the early stage as well, since there are indications in the literature that immunotherapy and treatment with TKIs could give early stage, surgical patients a survival benefit as well. [38, 39] In addition, as the management of acquired resistance mechanisms continues to evolve, patients could be treated with multiple lines of TKI treatment, which would further increase the number of testing moments.

1.6.1 A history of molecular testing

Since the discovery of the structure and function of DNA and specifically the Watson-Crick DNA model in 1953, molecular testing has taken off. In the 1980s, molecular testing became routed in routine diagnostics, via the application of Southern blotting to identify DNA alterations in Duchenne and fragile X syndrome.

In the 1993, the invention of polymerase chain reaction (PCR) [40] and the following introduction into clinical diagnostics was an important milestone, that enabled large-scale testing for HIV, hepatitis and other infectious diseases. In the late 1990s, genetic testing was applied to population screening for the first time, when testing newborns for cystic fibrosis mutations became the standard.

The first application of genetic testing for oncology occurred with the testing for the BRC:ABL translocation, the most common driver of chronic myelogenous leukemia (95%) and associated with acute lymphoblastic leukemia (ALL). With a novel and potent ABL TKI [41, 42] available – the first TKI ever described – all CML patients were required to undergo testing for the BRC:ALB fusion product, widely known as the ‘Philadelphia gene’. This testing was first implemented using Sanger sequencing, a widely used and relatively fast technique, [43] which was later also commonly used to detect the first pathogenic EGFR mutations, including p. L858R.

However, it was only after the completion of the Human Genome Project in 2003, that the focus of molecular diagnostics research truly shifted from infectious and hereditary diseases to cancer. With the approval of Herceptin for HER2-amplified breast cancer, testing for HER2 amplifications suddenly became a requirement, which led to the implementation of fluorescent in situ hybridization in many Pathology laboratories. [44] Additionally, the discovery of gefitinib-sensitizing EGFR mutations became an incentive for testing metastatic NSCLC for exon 19 deletions and L858R mutations in EGFR. [45]

Around the same time, PCR and automated Sanger sequencing were slowly replaced by next-generation DNA sequencing, a high-throughput multi-target method with wide applicability. In the mid-2010s, many laboratories started using DNA NGS to routinely screen for frequently occurring somatic oncogene mutations in KRAS, EGFR, BRAF, etc. [46] Although DNA NGS is well suited to find point mutations, small deletions, insertions and (when the tumor cell percentage is sufficient) amplifications, the identification of fusions and exon skipping events was always performed with in situ hybridization. In recent years, RNA NGS has replaced ISH in many laboratories.

The newest step in this rapid succession of molecular panels is the transfer to whole genome sequencing (WGS) and whole exome sequencing (WES). WGS and WES utilize massive sequencing panels covering the entire genome or exome. Although not yet routinely used for diagnostics in all clinical laboratories, WGS and WES are routinely applied for research purposes.

1.6.2 Current molecular testing methods

Testing methods currently available in routine diagnostic laboratories in the Netherlands include: immunohistochemistry (IHC), in situ hybridization (ISH), DNA NGS and RNA NGS. Each method has distinct advantages and disadvantages, as discussed below, which makes choosing the optimal workup challenging and complex.

1.6.2.1 Immunohistochemistry

IHC can be used to measure expression of a specific protein. (Figure 6A) In NSCLC, literature indicates that several genetic targets can be identified with IHC (ALK fusion, [47] ROS1 fusion, [48] NTRK fusion, [49] HER2 amplification [50]), as well as susceptibility to immunotherapy (PD-L1 expression). [51, 52]

The most important advantage of IHC is it's speed and price – IHC is a relatively fast and cheap method. A distinct downside is the fact that IHC is a single-target assay. In addition, IHC results can be subjective, and thus less than 100% sensitive and specific.

1.6.2.2 In situ hybridization

ISH can be used to identify translocation or amplification of a specific gene. (Figure 6B, 6C) In NSCLC, ISH is frequently used, to detect amplifications in MET, [53] EGFR [54] and HER2, [50] as well as fusions in ALK, [47] ROS1, [48] RET [55] and NTRK. [49] As ISH is a single-target assay, a separate analysis is required for each target gene, which is a disadvantage. In addition, information about the breakpoint and fusion partner is generally not provided by ISH. An important advantage over DNA NGS, however, is the ability to detect polysomy and amplifications in part of the tumor cells, which is an important benefit when dealing with intratumor genetic heterogeneity in TKI resistance cases.

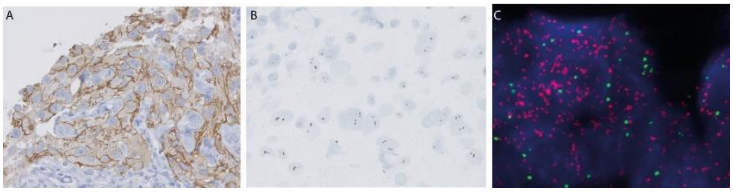

***Figure 6**: IHC and ISH examples. A: HER2 IHC. B: HER2 silver ISH. C: MET:C7 fluorescent ISH. Adapted from Chapter 3.*

1.6.2.3 DNA NGS

DNA NGS panels are able to identify alterations on a single nucleotide level within the DNA. DNA NGS can detect point mutations, small deletions and insertions with high accuracy. To some extent, DNA NGS also detects copy number variance, exon skipping and fusions. However, DNA NGS detections are highly dependent on which targets are included in the panel. Whole genome sequencing covers the entire genome, and is well suited to detect copy number variance, exon skipping and fusions, but also requires a much higher tissue input. Most laboratories therefore use a much smaller panel, which covers hotspots in cancer genes, but excludes introns and non-hotspot areas. Most clinically used DNA NGS panels therefore can't detect fusions and exon skipping. The ability to detect amplifications with smaller DNA NGS panels greatly depends on the tumor cell percentage. When the tumor cell

percentage is too low, amplifications can easily be missed, especially in tumors with intratumor genetic heterogeneity.

1.6.2.4 RNA NGS

RNA NGS identifies alterations on a single nucleotide level within the RNA. RNA NGS therefore identifies fusions, exon skipping events, point mutations and small insertions and deletions of covered regions. In contrast to ISH and IHC, DNA NGS and RNA NGS are multi-target assays. An important advantage of RNA NGS over ISH is the ability to detect fusion partners and breakpoint. Although RNA NGS uses more tissue than one ISH analysis, RNA NGS is a multi-target assay, meaning that multiple genes can be screened for translocations in one test. RNA NGS is less effective in bone lesions, as RNA quality decreases from the chemicals used in the decalcification process. The applications of RNA NGS and the techniques discussed above are summarized in Figure 7.

1.7 Immunotherapy

Immunotherapy is based on the interaction between programmed death ligand 1 (PD-L1) and programmed death 1 (PD-1). (Figure 8) When tumor cells express PD-L1, and this binds to the PD-1 receptor on T-cells, T-cells deactivate or undergo apoptosis, thus providing an effective method of immune evasion for the tumor. [56] Blocking this PD-1/PD-L1 interaction with monoclonal antibodies such as anti-PD-1 (nivolumab, pembrolizumab) constitutes an effective anti-tumor therapy, that has been reported to lead to a substantial survival benefit. [52, 57-60]

1.7.1 Immunotherapy, a brief history

The first mention of cancer immune therapy in the scientific literature was in 1891, when sarcoma and lymphoma patients were treated with live, inactivated Streptococcus pyogenes and Serratia marcescens by dr. Coley, in an attempt to activate the immune system against the tumor. [61, 62] Since then, the idea of activating the immune system and using it as a weapon against cancer, has taken root, and pan-cancer immunotherapy became a distant dot on the horizon. Since the discovery of the PD-1/PD-L1 interaction and its role in the immune evasion in cancer became clear in 2002, [56] that dot has become a lot closer. Years of murine models and preclinical testing

finally resulted in the approval of ipilumab in 2011 [63] and nivolumab [59, 64] and pembrolizumab in 2014 [58].

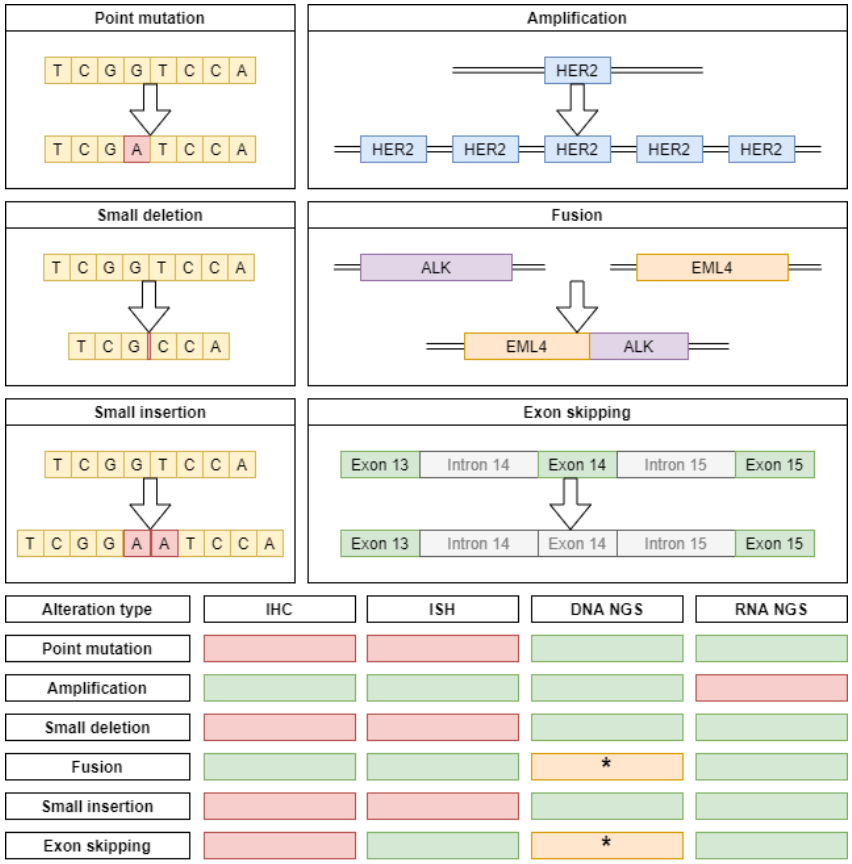

***Figure 7**: testing for molecular alterations per alteration type. Green rectangle: testing is possible; red rectangle: testing is not possible; orange rectangle: sometimes possible. *: only with good coverage of introns, for example whole genome sequencing (WGS).*

Being high-volume and high-mutational burden tumors, most of these immunotherapy regimens were registered for advanced metastatic NSCLC and melanoma fist, but are now expanding to other cancers as well, including breast cancer and head- and neck squamous cell carcinoma, with promising results. Immune regulatory drugs have opened up an entirely new avenue for cancer treatment and research, which is a true game changer for the field of Medical Oncology, and was thus awarded the Nobel Prize in 2018. [65]

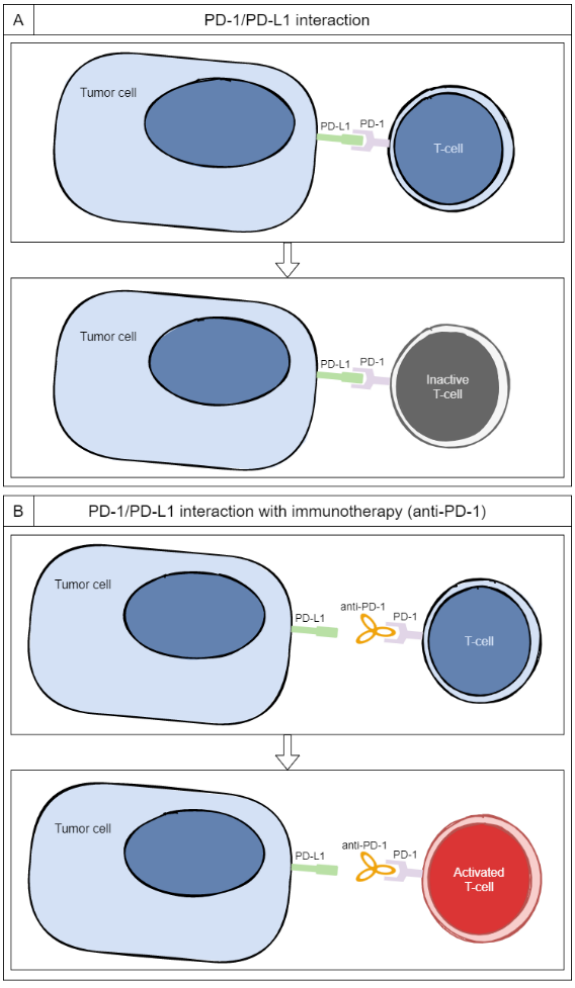

***Figure 8**: PD-1/PD-L1 interaction and immunotherapy. A: interaction between tumoral PD-L1 and T-cell PD-1, resulting in T-cell apoptosis. B: inhibition of the PD-L1/PD-1 interaction by immunotherapy (monoclonal anti-PD-1 antibodies, pembrolizumab or nivolumab), resulting in T-cell activation.*

1.7.2 Immunohistochemical PD-L1 as a biomarker for immunotherapy response

However, not all patients respond to immunotherapy to the same extent. Whereas some patients achieve durable progression-free survival, others have limited or no benefit from immunotherapy. [58, 59] The most important predictive biomarkers are the expression of immunohistochemical PD-L1 on tumor cell membranes [52, 58] and the tumor mutational burden (TMB), [66]

which have, since the introduction of immunotherapy in the clinic, become tremendously important in routine diagnostics.

However, both biomarkers are problematic. Both are non-perfect predictors of immunotherapy response: some patients with high PD-L1 expression or TMB fail to respond, and vice versa. In addition, PD-L1 is subject to substantial interobserver variance due to human limitations in estimating percentages, and there is a risk of sampling error, due to the intratumor heterogeneity of PD-L1 expression. (Formula 1) TMB can be assessed with multiple bioinformatics methods and NGS panels, each leading to a different 'mutations per megabase' score. Most of the currently used (lung) cancer panels are too small to determine the TMB, only the bigger panels (with higher drop-out) are able to, which means not all laboratories are able to assess TMB yet. And finally, due to intratumor genomic heterogeneity, (1.4.1) the TMB is not the same in all tumor regions, which could lead to sampling error.

$$PD-L1\ score = \frac{\text{PD}-\text{L1 positive tumor cells}}{\text{PD}-\text{L1 positive tumor cells} + \text{PD}-\text{L1 negative tumor cells}}$$

Formula 1: *PD-L1 tumor proportion score.*

1.7.3 Future biomarkers

Therefore, the search for new biomarkers for immunotherapy response continues. Most efforts have been focused on the makeup of the tumor immune microenvironment (TME), which is one of the key elements of an anti-tumor response. Important potential biomarkers, which have already shown to be associated with immunotherapy response in lung cancer are: tumor-infiltrating cytotoxic (CD8+) T-cells, [61, 67] M2-polarized macrophages, [67, 68] plasmablasts, [67, 69] IFNγ messenger RNA, [67, 70] dendritic cells [67, 71] and macrophage PD-L1 expression. [67, 72] In addition, there are numerous potential biomarkers correlated to immunotherapy response in other cancers, such as tertiary lymphoid structures. [67]

What this quantity of TME studies illustrates, is that the interaction between cancer and immune system is complex – perhaps even more so than we now realize. Efforts to compress this complexity into a single-target biomarker such as PD-L1 are ambitious but also slightly naïve. In the future, we might be able to comprehensively assess the TME and come with personalized and accurate

immunotherapy response predictions, but for now, patient selection remains imperfect.

1.8 Thesis Outline and Aims

To summarize, there are more treatment options for NSCLC than ever before, which makes selecting the treatment regimens an increasingly complex task. Pulmonary pathologists are faced with the difficult challenge of testing patients for a wide range of molecular alterations and predicting immunotherapy susceptibility. For this task, they can use a number of tests: IHC, ISH, DNA NGS and RNA NGS. However, in this rapidly changing field of molecular diagnostics and cancer immunology, the optimal testing method is not always clear. A balance must be found between tissue-efficiency, time, costs and comprehensiveness of testing.

The general aim of this thesis is therefore to retrospectively investigate the current testing landscape, and identify the most optimal testing sequence for NSCLC patients, at three key decision making moments:

1. Early stage NSCLC
2. Late stage NSCLC, treatment-naïve
3. Late stage NSCLC after acquired resistance

Chapter 2 describes the yield of molecular testing in early stage NSCLC. **Chapter 3** discusses the optimal workup in stage IV NSCLC. **Chapter 4** investigates the role of AI in PD-L1 immunoscoring. **Chapter 5** describes the sensitivity of NTRK IHC, and whether it should be used in routine diagnostics. **Chapter 6** describes the molecular workup after acquired resistance to EGFR TKIs.

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